

**EFFECTS OF CONTINUOUS TREATMENT WITH GONADOTROPIN-
RELEASING HORMONE DURING THE ANOVULATORY SEASON ON
GONADOTROPIN SECRETION, FOLLICULAR DYNAMICS AND
OVULATION IN THE MARE**

A Thesis

by

STEPHANIE MORTON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2004

Major Subject: Physiology of Reproduction

**EFFECTS OF CONTINUOUS TREATMENT WITH GONADOTROPIN-RELEASING
HORMONE DURING THE ANOVULATORY SEASON ON GONADOTROPIN
SECRETION, FOLLICULAR DYNAMICS AND OVULATION IN THE MARE**

A Thesis

by

STEPHANIE MORTON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Approved as to style and content by:

Gary L. Williams
(Chair of Committee)

Paul G. Harms
(Member)

Katrin Hinrichs
(Member)

John W. McNeill
(Head of Department)

December 2004

Major Subject: Physiology of Reproduction

ABSTRACT

Effects of Continuous Treatment with Gonadotropin-releasing Hormone during the Anovulatory Season on Gonadotropin Secretion, Follicular Dynamics and Ovulation in the Mare.

(December 2004)

Stephanie Morton, B.S., McNeese State University

Chair of Advisory Committee: Dr. Gary L. Williams

Objectives were to determine if low-dose, continuous infusion of GnRH from Fall to Spring, would prevent seasonal anovulation in mares. Twenty Quarter Horse mares, ages 18 mo to 24 yrs, were stratified by age and body condition score and assigned randomly to either a saline control (n = 9) or GnRH (n = 11) treatment group. Treatments were instituted between September 23 and October 9, 2002. Gonadotropin-releasing hormone was delivered in 0.9% physiological saline via Alzet osmotic minipumps (Model 2004) placed sc at the base of the neck, with Silastic sham pumps placed in control mares. Pumps were inserted on day 3 following ovulation or during the follicular phase if ovulation had not occurred. Delivery rate of GnRH was 2.5 ug/h (60 ug/d) for the first 60 d, followed by 5.0 ug/h (120 ug/d) thereafter, with all pumps replaced every 30 d. By December 1, all mares had become anovulatory and remained anovulatory until February. Mean serum concentrations of LH were not affected by treatment in anovulatory mares. In contrast, control mares that exhibited ovulatory cycles after treatment onset had higher ($P < 0.05$) mean concentrations of LH during all

phases of the estrous cycle except diestrus. Mean serum concentrations of FSH were not affected by treatment, but were lower ($P < 0.05$) from November through January relative to all other months in anovulatory mares. Interovulatory intervals in mares that cycled temporarily did not differ between groups. Ovulatory control mares had slightly larger ($P < 0.10$) follicles overall than GnRH-treated mares; however, ovulatory follicle diameters for control and GnRH-treated mares did not differ. Ovulatory control mares had higher ($P < 0.10$) mean concentrations of progesterone during metestrus and late diestrus. In a subgroup of control ($n = 5$) and GnRH-treated ($n = 5$) mares, total releasable pools of LH in response to 1 mg GnRH did not differ between groups. Ovulation resumed in 3 control and 3 GnRH-treated mares by March 30. Results indicate that continuous infusion of native GnRH at the doses employed herein is not sufficient to maintain ovulatory cycles during the anovulatory season.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. Gary L. Williams for his guidance and patience throughout my studies. His assistance with experiments and writing of this thesis were invaluable. Additionally, I would like to thank the members of the advisory committee, Dr. Paul Harms and Dr. Katrin Hinrichs, for their constructive participation during my studies.

Special thanks are extended to the employees of the Texas Agricultural Experiment Station, Beeville. Their hard work, long hours and dedication to my experiment is immensely appreciated. Additionally, I would like to thank Lloyd Vaughn and Flying V Quarter Horses for their cooperation and generosity in loaning twenty mares for this experiment.

Heartfelt appreciation is expressed to my parents, Steven and Janice Morton, for their unconditional support. Their encouragement and faith in my abilities gave me the will and fortitude to continually move forward and complete this degree. I have an undying admiration for their accomplishments in life and gather great strength from their example.

Finally, I thank Jared Collins, without whom this work would not have been completed. I value his kindness, patience and strength of character, as they were frequently tested. Additionally, I would like to thank him for his selflessness and encouragement throughout our many years together and as I continue to pursue my educational goals.

TABLE OF CONTENTS

	Page
ABSTRACT	iii
ACKNOWLEDGEMENTS	v
TABLE OF CONTENTS	vi
LIST OF FIGURES	viii
LIST OF TABLES	ix
 CHAPTER	
I INTRODUCTION.....	1
II LITERATURE REVIEW	4
Seasonal influence on endocrine control of reproduction	4
Artificial photoperiod and control of cyclicity.....	5
GnRH receptors.....	6
Secretory patterns of GnRH influence gonadotropin release.....	7
Administration of exogenous GnRH to enhance cyclicity	10
III PITUITARY AND OVARIAN RESPONSES OF QUARTER HORSE MARES TO CONTINUOUS INFUSION OF GONADOTROPIN- RELEASING HORMONE THROUGHOUT THE ANOVULATORY SEASON.....	16
Introduction.....	16
Materials and methods.....	17
Experimental animals	17
Experimental procedures	18
Anterior pituitary responses to a single injection of GnRH.....	19
Estrous behavior and ovarian morphology	20
Hormone analyses	20
Statistical analyses.....	21
Results.....	23
Ovarian morphology.....	23
Hormone analyses	27
Anterior pituitary responses to a single injection of GnRH.....	32

CHAPTER	Page
IV CONCLUSIONS	34
REFERENCES	40
APPENDIX	47
VITA	52

LIST OF FIGURES

FIGURE	Page
1 Mean follicle diameter of anovulatory mares averaged over two-week time periods throughout the study.....	25
2 Mean follicle diameter of mares exhibiting ovulatory cycles at the beginning and/or end of the study, relative to day of the estrous cycle	26
3 Mean serum concentrations of FSH in mares exhibiting ovulatory cycles at the beginning and/or end of the study, relative to day of the estrous cycle.....	29
4 Mean serum concentrations of LH in mares exhibiting ovulatory cycles at the beginning and/or end of the study, relative to day of the estrous cycle.....	29
5 Mean serum concentrations of progesterone in mares exhibiting ovulatory cycles at the beginning and/or end of the study, relative to day of the estrous cycle	30
6 Mean serum concentrations of LH and FSH in anovulatory mares throughout the 28-week experiment.....	31
7 Pooled mean serum concentrations of FSH in anovulatory control and GnRH-treated mares averaged over each month of the study	32
8 Mean serum concentrations of LH after a 1.0 mg iv injection of GnRH in a subgroup of control (n=5) and GnRH-treated (n=5) mares	33

LIST OF TABLES

TABLE	Page
1 Mean values of ovarian morphology in control and GnRH-treated mares on March 3, 2003	27

CHAPTER I

INTRODUCTION

Seasonal anovulation of mares is a limiting factor in controlled breeding programs. Because breed organizations have established January 1 as the universal birth date of foals, an operational breeding season has been imposed upon the horse that does not coincide with its natural breeding season. In the Northern Hemisphere, the natural breeding season for the mare is between April and October when days are long. Between the months of November and March, approximately 85% of mares will exhibit erratic cycles and/or cease ovulation (1). Gestation in the mare is approximately eleven months, requiring breeding to begin in February to produce competitive foals with birth dates near January 1. Therefore, the conflict between the universal birth date and seasonal anovulation of the mare burdens breeding operations, both managerially and economically.

Decreasing the period of anovulation is often required to facilitate efficient reproduction during the operational breeding season. Extending day length with artificial lighting to achieve a total of 14-16 h of light, beginning in mid winter, results in earlier onset of ovulation for a majority of mares (1-3). Although effective, the adoption of this practice is limited. Therefore, mares are often brought to breeding farms still undergoing transition from winter anovulation into the resumption of ovulatory cycles, resulting in extended boarding and handling for teasing and palpation. This increases

This thesis was written in the style and format of Theriogenology.

the cost of management to the breeder and owner. Effective and efficient methods of controlling seasonal anovulation are needed to minimize economic losses associated with failure to establish normal estrous cycles during the operational breeding season.

Recent efforts to control seasonal anovulation have focused on the administration of exogenous gonadotropin-releasing hormone (GnRH) and its analogues (GnRHa). The ability of exogenous GnRH to influence ovarian cyclicity is dependent upon an adequate number of GnRH receptors on gonadotrophs of the anterior pituitary and the type of releasing hormone administered. The mare is unique in her ability to maintain a high concentration of GnRH receptors during both seasonal anovulation and during continuous treatment with exogenous GnRH (4, 5). Analogs of GnRH differ in their amino acid sequences relative to native GnRH, and these differences can either increase or decrease their biological activity. Increased receptor binding affinity is an effect observed with most GnRHa, regardless of whether the analog acts as an agonist or antagonist (6). Although several potent GnRHa have been used to stimulate gonadotropin secretion in anovulatory mares, ovarian responses have been highly variable and often disappointing. Several studies indicate that administration of native GnRH at low doses administered to seasonally anovulatory mares may be successful in stimulating positive pituitary responses, and thus ovulation (7-10). Collectively, these reports have led us to hypothesize that continuous low-dose infusion of GnRH, administered to cyclic mares beginning in October (Northern Hemisphere) and continuing through the winter, will prevent the onset of seasonal anovulation. Objectives of the current study were to test that hypothesis, and to determine hormonal

profiles, estrous behavior, interovulatory intervals, and ovarian characteristics associated with GnRH treatment.

CHAPTER II

LITERATURE REVIEW

Seasonal influence on the endocrine control of reproduction

The mare is a seasonally polyestrous animal whose estrous cycles are regulated by photoperiod. During periods of decreasing day length, gonadotropin secretion declines and 70-85% of mares in the Northern Hemisphere cease ovulation (1, 11). As day length increases, the mare undergoes a period of transition in which the hypothalamic-pituitary axis becomes reactivated and increasing amounts of gonadotropins are secreted (2, 5). This transitional period is associated with erratic periods of estrus, which are typically infertile because of a lack of follicular development and ovulation (1). The natural ovulatory season for the mare in the Northern Hemisphere is between April and October. The majority of mares will cease ovarian activity at some point during the period from November to March.

Seasonal anovulation in the mare is a direct effect of day length as perceived by the eyes and signaled via a multineuronal pathway to the pineal gland. Dusk, as perceived by the animal, is associated with a sharp increase in melatonin secretion from the pineal gland. An increase in melatonin release persisting for 10 h after dusk defines a long night (12). Although the mechanisms are unknown, melatonin reduces gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus. Hypothalamic and pituitary contents of GnRH and luteinizing hormone (LH), respectively, fluctuate with season or photoperiod and are lowest during the middle of

the anovulatory season (13). However, content of follicle stimulating hormone (FSH) in the pituitary exhibits no seasonal change (5, 13). This implies that the decrease in GnRH secretion reduces the synthesis of LH, but not FSH, in the seasonally anestrous mare. In contrast, the ewe, which is a short-day breeder, has a positive hypothalamic and pituitary response to increases in melatonin (14). Unlike the mare and ewe, cows do not typically experience cessation and re-establishment of cyclic ovarian activity in response to changes in photoperiod (14).

Artificial photoperiod and control of cyclicity

The universal birth date (January 1) imposed by many breed organizations has caused a misalignment of the operational and natural breeding season of the mare. Current management practices designed to control seasonal anovulation and enhance early onset of estrous cycles include artificial lighting programs and use of exogenous hormones (1, 15). Although sheep are seasonal breeders, lighting regimens are not practical for this species because exposure to light must be reduced to induce cyclic activity. In contrast, artificial lighting programs for horses are designed to increase day length in the fall and winter months prior to the breeding season. By removing the stimulus for melatonin secretion, the long night, mares will exhibit increased gonadotropin secretion and the onset of normal estrous cycles can be hastened (1, 2). By providing 14-16 h of total daylight beginning in early December, the majority of mares will establish normal estrous cycles near March 1 (1, 15). Artificial light should be added at the end of the day, prior to dusk, postponing the sharp increase of melatonin and preventing the presence of melatonin 10 h after dusk (1, 12). Artificial lighting

results in a measurable increase in LH pulse frequency with decreasing amplitude, thereby enhancing the potential for resumption of ovulation (2). Lighting programs, when properly managed, effectively establish early, fertile estrous cycles in many mares (1). However, many horse owners and breeders do not use lighting programs due to their inconvenience of application. Therefore, alternative methods for management of seasonal anovulation would be well-received.

GnRH receptors

Reproduction in mammals is controlled via the hypothalamic-hypophyseal axis. The interaction between the hypothalamus and anterior pituitary is dependent upon the secretion of releasing hormones from the hypothalamus and the presence of their receptors on the pituitary. Specifically, gonadotropin-releasing hormone receptor concentration and affinity are principal factors regulating the synthesis and release of LH and FSH from the anterior pituitary, and thus ovulation. Castration, steroid hormone concentrations, and fluctuating reproductive states influence receptor number and affinity. Additionally, endogenous GnRH is an important up-regulator of its own receptor (16). The physiological increase in estradiol prior to the LH surge also enhances GnRH receptor concentration and affinity in all species studied (16). Anterior pituitary receptors for GnRH are maintained in the mare in relatively high numbers throughout all seasons (5, 13). Porter and Sharp (4) recently characterized GnRH receptor trafficking in the mare. They observed a slow rate of receptor endocytosis compared to that reported for other species, as well as increased GnRH receptor binding in the presence of continuous GnRH infusion. When pituitary GnRH receptor

concentrations were measured in cycling heifers, concentrations during estrus and the follicular phase were higher compared to the luteal phase of the estrous cycle (17). Non-cyclic and pregnant cows exhibited the lowest concentrations of GnRH receptors compared to other physiological states (17). Tortenese et al. (18) has shown that gonadotroph cell populations are affected by season and stage of estrus in the mare. A significant increase in density of gonadotrophs in the pars tuberalis occurred in cyclic mares during both the breeding and non-breeding seasons as compared to seasonally anestrus mares. Similar studies in the ewe revealed no seasonal effects on gonadotroph cell populations in the pars tuberalis or pars distalis and noted a lower density of cells compared to the horse (19). These studies indicate that density of gonadotroph cell populations may, in part, motivate the seasonal changes in gonadotropin release in the mare. Additionally, when a decline in GnRH receptor concentration, affinity, and/or sensitization occurs, a decrease in LH release is observed in cattle (17), horses (20), sheep (21), and rodents (16). The limited availability of LH for release is considered the primary cause of anovulation in the mare. However, the ability of the equine anterior pituitary to maintain GnRH receptor affinity and concentration throughout the anovulatory season implies that exogenous GnRH can be used successfully to control seasonal anovulation.

Secretory patterns of GnRH influence gonadotropin release

Patterns of GnRH and gonadotropin secretion are highly correlated during all stages of the estrous cycle (14, 22-25). In the diestrus mare, concurrent pulses of LH and FSH measured in the periphery are accompanied by hypothalamic release of GnRH

(22). However, small pulses of gonadotropin release can be detected in venous blood of the intercavernous sinus between major pulses of GnRH, and contribute to the basal level of circulating hormone (23). Intercavernous sinus samples collected every 30 sec from mares in estrus revealed a virtually continuous release of GnRH, with larger pulses occurring every 30-60 min (22). The latter were accompanied by an LH and FSH pulse. Research conducted by Irvine and Alexander also demonstrated that about 35% of low amplitude pulses of GnRH are not associated with a pulse of LH or FSH (23). A similar study using mares during the periovulatory period reported 9% of low amplitude GnRH pulses failed to initiate a pulse of LH or FSH (24). These observations suggest that low amplitude pulses of GnRH may stimulate gonadotropin synthesis and/or release at low amplitudes, while high amplitude pulses of GnRH stimulate high amplitude pulses of LH and FSH. This may explain why various GnRH treatments successfully stimulate the release of LH and FSH in sufficient amounts and induce ovulation in the mare. However, exogenous administration of GnRH to humans, sheep, and rodents must be pulsatile to mimic the endogenous patterns of GnRH and to avoid receptor desensitization or down-regulation (25).

Prepubertal heifers given GnRH for 72 h in a pulsatile (2.5 ug/2 h) or continuous (1.25 ug/h) pattern responded initially with an increase in serum concentrations of LH and estradiol (26). However, continuous infusion ultimately resulted in a delay in puberty compared to pulsatile infusion. The delay in puberty was a result of receptor down-regulation or desensitization of the pituitary to endogenous GnRH and loss of LH pulsatility (27). Gong et al. (28) reported an initial increase in serum LH and FSH

following osmotic pump infusion of buserelin, a GnRH analogue (GnRHa), for 48 d. However, circulating concentrations returned to baseline by day 8 of treatment and remained low until 4-5 d after pump removal. The same laboratory produced similar results when 5 or 10 ug of buserelin were administered i.m. twice daily for 3 weeks (29). After an initial increase in serum concentrations of LH, the gonadotropin returned to basal levels, follicles failed to develop beyond 9 mm, and both the LH surge and ovulation failed to occur. These and other similar data have led to the experimental use of GnRH and GnRHa as management tools for suppressing reproductive cyclicity in cattle (30).

A comparative study involving pony mares and ewes provided stark contrasts of responses to pulsatile and continuous infusion of GnRH. Pony mares responded to all GnRH treatments with increased plasma concentrations of LH (25). In contrast, ewes treated continuously with GnRH (2.5 and 25 ug/h) had reduced concentrations of LH and reduced pulse frequency, indicative of receptor down-regulation. However, ewes receiving 250 ng/h in a pulsatile fashion exhibited patterns of LH secretion that did not differ from controls (25). In another study, pulsatile infusion of GnRH (250 ng/2h) to nutritionally restricted ewes was successful in restoring gonadotropin synthesis and secretion to levels similar to that of normally-fed controls (31). In congenitally GnRH-deficient, hypogonadal mice, chronic pulsatile infusion of GnRH stimulates receptor up-regulation and rapid increases in synthesis and release of FSH and LH, with the rate slower for LH than FSH (16). A study in amenorrheic women compared ovarian responses to pulsatile and continuous infusion of GnRH (32). All women ovulated

during the pulsatile regimen and had significantly increased serum concentrations of FSH, LH and estradiol compared to controls. However, continuous infusion produced no ovulations and no significant rise in biologically active gonadotropin or serum concentration of estradiol. These observations demonstrate the importance of pulsatile infusion of GnRH in humans, sheep and rodents for producing a positive gonadotropic response. However, cattle exhibit greater sensitivities to GnRH and GnRHa treatments, resulting in down regulation of receptors and loss of LH secretory patterns sufficient to induce cyclicity or ovulation. Collectively, the published literature supports the idea that the mare is unique in her ability to respond to long-term, continuous treatment with GnRH.

Administration of exogenous GnRH to enhance cyclicity

Although mechanisms for positive responses to long-term exposure of exogenous GnRH are present in the mare, type of GnRH preparation, dose, and method of administration have proven to be sources of significant variation relative to expected responses. Multiple studies utilizing the GnRHa, goserelin, in seasonally anovulatory mares during February (August, Southern Hemisphere) provided variable results. Continuous infusion using a sc implant that delivered 60 ug/d or greater of goserelin for 28 d (7, 32, 33) resulted in mares exhibiting variable LH, FSH and ovulatory patterns. Fitzgerald et al. (33) observed 61% of mares receiving 60 ug/d of goserelin ovulating in year 1, but only 11% ovulated in response to the same dose in year 2. Frequency of ovulation decreased in mares receiving higher concentrations of goserelin overall, with variation between years for identical treatments. Turner and Irvine (7) reported that

continuous infusion of 120 ug/d of goserelin resulted in 54% of mares ovulating between days 5 and 19 of treatment and developing a functional corpus luteum. However, 18% of mares ovulated between days 19 and 27 and failed to develop a functional corpus luteum. The remaining mares failed to ovulate during treatment, but all ovulated within 3 weeks after the end of treatment (7). Concentrations of LH and FSH during the periovulatory period exhibited normal patterns for mares that ovulated and developed a corpus luteum. Mares which ovulated but failed to establish a corpus luteum exhibited concurrent LH and FSH peaks during the peri-ovulatory period (7). Mumford et al. (34) reported ovulation in only 10% of mares receiving continuous infusion of 60 ug/d of goserelin. Concentrations of LH peaked at day 12 of treatment and increased with increasing doses of goserelin. Those mares with follicles ≤ 15 mm in diameter at the start of treatment ovulated less frequently than those with follicles 16 to 20 mm in diameter regardless of treatment group. Importantly, of the mares that ovulated in all treatment groups, the mean interval to the next ovulation was 71 d (34). All reports have indicated an inconsistency in repeatability of treatment responses, with stage of follicular development at the start of treatment playing an important role in their success. Additionally, the extended interovulatory interval reported by Mumford et al. (34) suggests down-regulation of GnRH receptors in the pituitary of mares receiving goserelin, resulting in decreased gonadotropin secretion and failure of ovulation.

A comparison of pulsatile GnRH with GnRHa (buserelin) given twice daily in anestrus mares from January to March resulted in GnRH-treated mares ovulating (11/14) during all months (8). Mares treated with GnRH were given a 10 ug pulse every

hour administered over a 1-min period using a peristaltic pump, with GnRHa-treated mares given one 10 ug injection twice daily. The GnRHa was not effective in inducing ovulation during January. All mares that ovulated in January and February returned to anestrus when GnRH or GnRHa support was removed (8). Additionally, the GnRH-treated group had higher concentrations of LH, regardless of the occurrence of ovulation, than did the GnRHa-treated group. Failure of GnRHa-treated mares to ovulate in January indicates an inadequate degree of pituitary stimulation due to dose or method of administration. In this study, twice daily injections may have been insufficient to produce a physiological pattern of LH secretion and concentration effective for inducing ovulation in deeply anestrus mares. Alternatively, the GnRHa dose may have been too high, resulting in GnRH receptor down-regulation. However, the pulsatile administration of GnRH provided adequate pituitary stimulation regardless of photoperiod or stage of anestrus.

Due to extreme differences in potency and action on GnRH receptors between native GnRH and GnRH analogues, several studies have focused on the use of native GnRH to treat seasonally anovulatory mares. Methods of administering native GnRH have also been compared using pulsatile iv (9, 10), continuous iv (10), or continuous sc infusion (35, 36). Johnson reported that administration of GnRH by pulsatile infusion at dosages of 2 and 20 ug/h resulted in ovulation within 5.7 and 12 d, respectively (9). However, the occurrence of multiple ovulations was high, ranging from 1-6 in small groups (n=7) of mares receiving 20 ug/h of GnRH. A similar experiment from the same laboratory compared pulsatile and continuous iv infusion of GnRH at the same doses

(10). Mares receiving pulsatile treatment had earliest days to ovulation and highest concentrations of LH on the day of ovulation compared to control and continuously infused mares. However, these mares had smaller follicles and an increased incidence of multiple ovulations compared to mares receiving continuous infusion of GnRH at 2 and 20 ug/h (10). Mares receiving continuous iv infusions of GnRH at 2 ug/h (4/4) failed to ovulate spontaneously within 16 days of treatment, but did ovulate after an injection of 2,000 IU of hCG with mean follicle size of 49 mm. Multiple studies from Australia have demonstrated the use of continuous infusion of GnRH in mares in seasonal anovulation and mares undergoing transition from seasonal anovulation into the breeding season. Hormone was delivered at a rate of 45 (Group 1) or 90 (Group 2) ug/h via osmotic minipump for 28 d (35). Treatment resulted in fewer days to ovulation for mares treated with GnRH in Group 1 (19.3 ± 1.6) and Group 2 (15.9 ± 1.5) compared to controls (34.2 ± 2.5). The percentage of mares that ovulated within 35 d of onset of treatment was 90.5, 97.2, and 59.2% for Group 1, Group 2, and controls, respectively. Mean concentrations of LH were significantly higher in GnRH-treated mares compared to controls, but there was no difference between GnRH-treated groups (35). Mares undergoing transition were treated in a similar fashion using continuous sc infusion of GnRH at a rate of 45 ug/h (36). Days to ovulation were 18.6 and 54.8 d for treated and control mares, respectively. Additionally, volume of the ovulatory follicle doubled within 7 d for treated mares, while follicles of control mares increased only slightly. Plasma concentrations of LH rose to ovulatory levels within 5 d of the start of treatment for GnRH-treated mares, whereas an increase in LH for most control mares occurred

after day 40 (36). These data further demonstrate the ability of low-dose, continuous infusion of GnRH to enhance LH synthesis and secretion and to induce ovulation in seasonally anovulatory and transitional mares.

Results of administering GnRH to ovulatory mares during the breeding season supports the view that exogenous, native GnRH can be used in ways that do not markedly interfere with endogenous patterns of LH secretion (10, 37). Becker and Johnson reported that pulsatile and continuous iv infusion of GnRH at a rate of 20 ug/h to cyclic mares had no effect on the day of ovulation (10). However, mares receiving continuous infusion beginning on day 16 of the estrous cycle did have lower serum concentrations of LH on the day of ovulation compared to the pulsatile and control groups (10). In comparison, pulsatile iv infusion did not produce elevated serum LH concentrations compared to controls. These data suggest that 20 ug/h of GnRH administered to cyclic mares by continuous iv infusion may cause some down-regulation of the GnRH receptor and reduction of LH secretion without interfering with ovulation. However, pulsatile iv infusion at the same dose fails to stimulate the receptor and elevate LH concentrations in the cyclic mare.

Recent work conducted in our laboratory demonstrated the use of continuous sc infusion of GnRH at a rate of 2.5 ug/h (60 ug/d) to induce follicular development in idiopathic and lactationally anestrous mares during the physiological breeding season (37). Treatments were administered over three 14-d periods. Period I compared GnRH-treated mares (GnRH/GnRH) to untreated control mares (Control/GnRH). Mares failing to develop a 35 mm follicle or ovulate at the end of Period I were either switched to

GnRH treatment (Control/GnRH) or reimplanted (GnRH/GnRH). At the end of Period I, 47.8% (11/23) of GnRH-treated mares and 12.5% (2/24) of Control mares developed a 35 mm follicle and ovulated (37). Control mares receiving GnRH implants during Period II exhibited a marked increase in follicular development and ovulation. By the end of Period III, the percentage of ovulations was similar for both treatment groups at approximately 85% (37). Serum concentrations of LH increased for GnRH-treated mares. Additionally, administration of GnRH at this low dose to cyclic mares had no adverse effects on cyclicity or interovulatory interval (37). Results indicate that the equine pituitary can respond effectively to continuous infusion of low doses of native GnRH during both the anestrus and transitional periods.

CHAPTER III

PITUITARY AND OVARIAN RESPONSES OF QUARTER HORSE MARES TO CONTINUOUS INFUSION OF GONADOTROPIN-RELEASING HORMONE THROUGHOUT THE ANOVULATORY SEASON

Introduction

In the Northern Hemisphere, the natural breeding season for the mare is between April and October when daylength is long. Between the months of November and March, approximately 85% of mares will exhibit erratic cycles and/or cease ovulation (1). Because most breed organizations have established January 1 as the universal birth date of foals, an operational breeding season has been imposed upon the horse that does not coincide with its natural breeding season. Effective and efficient methods of controlling seasonal anovulation are needed to minimize economic losses associated with failure to establish normal estrous cycles during the operational breeding season.

Recent efforts to reduce the period of seasonal anovulation have focused on the administration of exogenous GnRH and its analogues (GnRHa). The ability of exogenous GnRH to influence ovarian cyclicity is dependent upon an adequate number of GnRH receptors on gonadotrophs of the anterior pituitary and the type of releasing hormone administered. The mare is unique in her ability to maintain a high concentration of GnRH receptors during both seasonal anovulation and during continuous treatment with exogenous GnRH (4, 5). Although several potent GnRHa have been used to stimulate gonadotropin secretion in anovulatory mares, ovarian

responses have been highly variable and often disappointing. Several studies indicate that low doses of native GnRH administered to seasonally anovulatory mares may be the most successful in stimulating positive pituitary responses, and thus ovulation (7-10). Collectively, these reports have led us to hypothesize that continuous low-dose infusion of GnRH, administered to cyclic mares beginning in October (Northern Hemisphere) and continuing through the winter, may have the potential to prevent the onset of seasonal anovulation. Objectives of the current study were to test that hypothesis, and to determine hormonal profiles, estrous behavior, interovulatory intervals, and ovarian characteristics associated with GnRH treatment administered in this manner.

Materials and methods

Experimental animals

Twenty non-pregnant Quarter Horse mares obtained in mid August 2002 were maintained on pasture, with free access to water and salt. Grain (14% crude protein, mixed grain, soybean meal and molasses; Falls City Milling, Falls City, TX) was provided as needed to adjust and maintain a body condition score of 5 (good; 1-9 scale). All mares were stratified by age and initial body condition score, then assigned randomly to a 1) GnRH-treated (n=12) or 2) Control (n=10) group. Mares ranged in age from 18 months to 24 years at the start of the experiment. Transrectal ultrasonography was employed to examine ovarian morphology and to confirm cyclicity prior to the start of the experiment. Mares were classified as cyclic upon visual confirmation of a corpus luteum. Young mares were assumed to have entered puberty based on age if cyclicity could not be confirmed. To aid with data collection, some estrous cycles were loosely

synchronized with PGF-2 α during the mid-luteal phase of a cycle before the start of the experiment.

Experimental procedures

Gonadotropin-releasing hormone was delivered in 0.9% physiological saline via an Alzet osmotic minipump (Model 2004; Durect Corporation, Cupertino, CA). Alzet pumps were incubated in 0.9% physiological saline at 37°C for 40 h prior to implantation as recommended by the manufacturer. Sham pumps were made from silicone tubing and filled with silicone to approximate the size of the Alzet pumps, then sterilized prior to surgical insertion. Beginning September 23 and continuing through October 9, 2002, GnRH and sham pumps were inserted on day 3 following a synchronized ovulation or during the follicular phase if ovulation had not occurred. Pumps were inserted surgically at the base of the neck, in front of the shoulder using aseptic technique. The surgical site (approximately 6 x 6 cm) was clipped free of hair, scrubbed with 7.5% providone-iodine scrub, and disinfected with 1.0% providone-iodine solution. Three to five cc of 2% Lidocaine HCl (Vedco, St. Joseph, MO) were injected sc and a 1 ½ - 2 cm incision was made through the skin using a no. 10 blade. A blunt surgical instrument was utilized to create a sc pocket ventral to the incision to accommodate the pump. Pumps were disinfected in a 2% chlorhexide gluconate solution (Nolvasan) and placed under the skin below the opening of the incision. The incision was closed using a non-absorbable suture (Suture Vet, Vetus Animal Health, Rockville Centre, NY) that was removed 7-10 days following the procedure. Pumps were removed utilizing the same procedure and a new GnRH or sham pump was inserted

every 30 days at alternating sites on both sides of the neck until the end of the experiment (March 31, 2003). During the first 60 d, GnRH-treated mares received GnRH (Bachem California Inc, Torrance, CA) at a rate of 2.5 ug/h (60 ug/d) followed by a delivery rate of 5.0 ug/h (120 ug/d) for the remainder of the experiment (approximately 120 days).

Blood samples were collected via jugular venipuncture to determine circulating concentrations of LH, FSH, and progesterone. When a follicle ≥ 35 mm was present or at the onset of estrus, whichever occurred first, blood was collected once daily until 3 days after ovulation. Blood samples were collected once every 2-3 days at all other times. Samples were placed on ice immediately following collection, then allowed to clot at room temperature for 2 h. Serum was harvested by centrifugation, divided into two aliquots, and stored at -20°C until hormone analyses.

Anterior pituitary responses to a single intravenous injection of GnRH

On March 5, 2003, 10 mares were selected from GnRH (n=5) and control (n=5) groups to determine if stores of LH in gonadotrophs had been affected by GnRH treatment. To avoid the potential for induced ovulation, only mares with all follicles less than 25 mm were used. Each mare was fitted with an indwelling jugular catheter (14 GA x 83 mm, Angiocath; Becton Dickinson, Sandy, Utah) using aseptic technique on the morning of the experiment. Mares were separated in pairs into small pens and allowed to rest for 1 h before the start of the experiment. Blood samples (10 ml) were collected at -60, -30, 0, 15, 30, 45, 60, 90, 120, 180, 210, and 240 min relative to iv injection of GnRH (1.0 mg in 2.0 ml 0.9% physiological saline) administered via the

catheter. Blood samples were dispensed into tubes containing 150 ul of solution containing heparin (1000 IU/ml) and 5% EDTA and placed on ice. All jugular catheters were removed at the end of the 5-h experiment.

Estrous behavior and ovarian morphology

Mares were teased in groups of five in a large pen adjacent to a stallion on a daily basis. Teasing scores were as follows: 1) breaks down, urinates, 2) winking, intense interest in stallion, 3) some interest in stallion, 4) passive, 5) rejects stallion. Palpation and transrectal ultrasonography (Concept/MCV, Dynamic Imaging, Livingston, Scotland, UK) were employed at 1-3 day intervals to assess ovarian morphology. When a follicle greater than or equal to 35 mm was observed or at the onset of estrus, whichever occurred first, mares were examined daily by transrectal ultrasonography until ovulation. Visual presence of a corpus luteum after ovulation was also confirmed by transrectal ultrasonography. An intensive comparison of ovarian morphology was conducted on March 3, 2003; all visible follicles from all mares in the study were counted and measured in mm using transrectal ultrasonography.

Hormone analyses

Serum concentrations of LH and FSH were determined by double antibody RIA for all samples collected. A highly purified equine LH (eLH AFP-5130A) preparation was used for both iodinated tracer and standards. An anti-eLH antiserum (AFP-240580) was used at a dilution of 1:120,000, which yielded 25.8% binding at B/O. A highly purified equine FSH (eFSH AFP-5022B) preparation was used for both iodinated tracer and standards. An anti-eFSH antiserum (AFP-2062096) was used at a dilution of

1:12,500, and yielded 29.4% binding at B/O. The intraassay and interassay coefficients of variation (CVs) were 7.9% and 5.5% respectively for LH, and 5.8% and 2.3% respectively for FSH assays. Serum progesterone was measured using a commercial single antibody RIA kit (Diagnostic Products Corporation, Los Angeles, CA). When mares had progesterone values below 1 ng/ml and minimal follicular activity only 2 samples per week were assayed. The intraassay and interassay CVs for progesterone were 10.3% and 4.1% respectively.

Statistical analyses

After the experiment was conducted, data from 2 mares, 1 control (#4090) and 1 GnRH-treated (#3025), were removed due to extreme variations in hormone levels and cycle characteristics. Mare 4090 had serum concentrations of LH (range of 6.25 to 31.70 ng/ml) greater than 2 standard deviations above the mean for all other mares for more than 60 days from the start of the experiment. Serum concentrations of progesterone were also elevated with values above 20 ng/ml for more than 30 days and not declining to < 1 ng/ml until late November. Mare 3025 (GnRH) was eliminated due to elevated serum concentrations of LH (range of 11.07 to 19.09 ng/ml) which were 2 standard deviations above the mean for the first 30 days of the experiment. This mare had interovulatory intervals in 5 of 8 cycles that were less than 20 days, 4 of which were less than 15 days. These short interovulatory intervals were not diestrous ovulations, as a decline in serum progesterone to levels below 1 ng/ml occurred prior to each ovulation. The final number of mares used for statistical analysis in control and GnRH-treated

mares was 9 and 11, respectively. However, one GnRH-treated mare died in mid January, but data collected until that time was included in all analyses.

Statistical analyses of estrous cycle variables included 1) interovulatory interval, 2) size of ovulatory follicles, and 3) number of ovulations per estrous cycle. Mares were classified as ovulatory or anovulatory based on serum concentrations of progesterone and visual confirmation of corpora lutea by transrectal ultrasonography.

Retrospectively, when progesterone had been less than 1 ng/ml for at least 30 days and no follicles greater than 30 mm had developed, mares were considered anovulatory. Once ovulation had resumed in an anovulatory mare, all data from that time forward was classified as ovulatory.

The PROC GLM procedure of SAS was employed to conduct repeated measure analysis of variance on reproductive and hormonal variables. The statistical model included the following sources of variation: Treatment, mare(treatment), time and treatment x time. Main effects of treatment were tested using mare(treatment) as the error term. When a significant F-value was obtained, means were contrasted using Bonferoni's T-test. Chi-square analysis was used for proportional data (i.e., cycling vs. not cycling). Areas under the curve were calculated for the 4 h period following the GnRH challenge and compared using the PROC T-TEST procedure. Numbers of small and medium follicles and average sizes of follicles from measurements determined on March 3, 2003 were contrasted using the PROC T-TEST procedure.

Results

Ovarian morphology

By the end of November, only 3/9 control and 3/11 GnRH-treated mares continued to exhibit ovulatory cycles. During the months of December through February, all mares were anovulatory. By the end of the experiment on March 31, 2003, 3/9 control and 3/11 GnRH-treated mares had resumed ovulation. Analysis of ovulation frequencies for each month revealed no differences between treatment groups. Diestrous ovulations occurred in 2/9 control mares and 0/11 GnRH-treated mares. One control mare ovulated in October and the other in November. There were no double ovulations during the treatment period in either control or GnRH-treated mares.

Interovulatory intervals for mares ovulating during the experiment ranged from 15 to 24 d, with means of 21.0 ± 1.0 and 21.3 ± 0.7 d for control and GnRH-treated mares, respectively. Mares that became anovulatory after the start of the study and resumed ovulation by March 31 had mean interovulatory intervals of 130.7 ± 15.2 d (control) and 154.3 ± 10.9 d (GnRH-treated), respectively. Anovulatory control and GnRH-treated mares that failed to ovulate by March 31 (end of study), had been anovulatory an average of 162.7 ± 15.1 and 155.6 ± 8.5 d, respectively.

Follicle measurements (mm) obtained throughout the experiment were compared for ovulatory and anovulatory mares within treatment groups. Follicles were divided into three categories A) less than 21 mm, B) 21 – 34 mm and C) greater than 34 mm. All mares had at least one follicle less than 21 mm (category A) present throughout the duration of the experiment. Follicle measurements greater than 21 mm were recorded

and used for analyses. The occurrence of follicles in categories B and C did not differ between treatment groups over the duration of the experiment. Additionally, there was no difference in occurrence of follicles in categories B and C between treatment groups for ovulatory and anovulatory mares throughout the experiment. However, the mean follicle diameter in mares after the onset of anovulation, averaged over 14 two-week periods, was larger ($P < 0.05$) in GnRH-treated mares compared to controls during periods 9 and 10 (Figure 1). Control mares had greater mean follicle diameter ($P < 0.05$) during period 11 than GnRH-treated mares. These periods were during the months of January and February. Treatment had no effect on follicle size in anovulatory mares at all other time periods. Mean follicle diameter of mares exhibiting ovulatory cycles at the beginning and/or end of the study was assessed within phase of the estrous cycle (Figure 2). Control mares had slightly larger ($P < 0.10$) follicles overall than GnRH-treated mares. Mean follicle diameter on the day of ovulation was 40.9 ± 1.45 (control) and 39.0 ± 0.98 mm (GnRH).

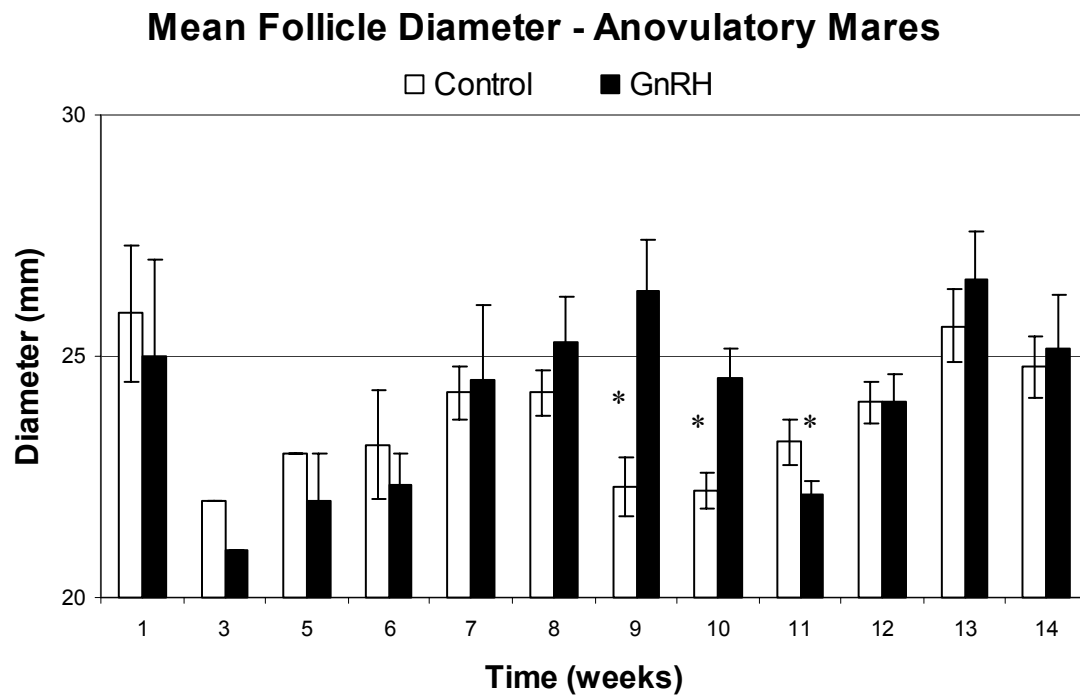


Figure 1: Mean follicle diameter (mm \pm SEM) of anovulatory mares averaged over two-week time periods throughout the study. * Means differed between treatment groups ($P < 0.05$). Treatment had no effect on follicle size at all other time periods.

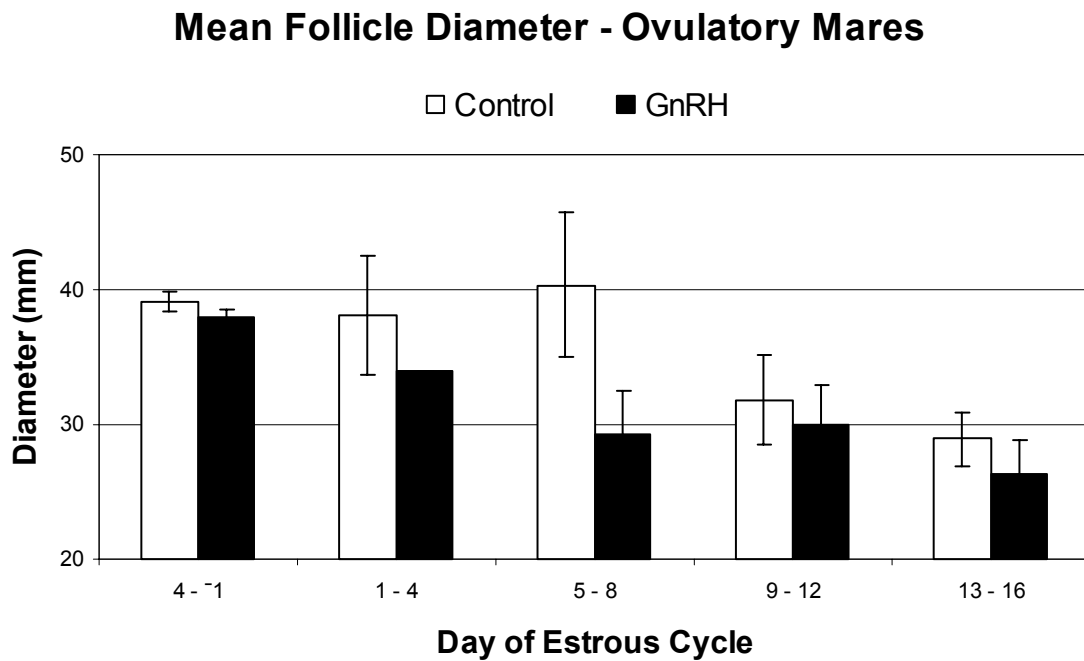


Figure 2: Mean follicle diameters (mm \pm SEM) of mares exhibiting ovulatory cycles at the beginning and/or end of the study, relative to day of the estrous cycle. Control mares had slightly larger follicles over all periods ($P < 0.10$).

On March 3, 2003, all mares were subjected to a comprehensive evaluation of all visible follicles using transrectal ultrasonography (Table 1). Follicles were separated into the following two categories based on mean diameter (mm) of an individual follicle: A) less than 15 mm and B) 15-30 mm. Average mean diameter of follicles in categories A and B did not differ between treatment groups, and 66.8% of all follicles were less than 15 mm in diameter (category A) on this date. Neither, the total number of follicles

present in individual categories (A and B) nor in both categories combined differed between control and GnRH-treated mares.

Table 1: Mean values of ovarian morphology in control and GnRH-treated mares on March 3, 2003. (Mean \pm SEM)

Treatment	No. of Follicles		No. of Follicles/mare (mean no. \pm SEM)		Diameter (mm \pm SEM)	
	A	B	A	B	A	B
Control (n=9)	72	36	8.0 \pm 1.0	5.1 \pm 1.0	11.0 \pm 0.3	18.5 \pm 0.6
GnRH (n=10)	65	35	6.5 \pm 0.8	4.9 \pm 1.3	10.2 \pm 0.4	17.9 \pm 0.9

Group A = follicles less than 15 mm and Group B = follicles 15 – 30 mm.

Treatment groups did not differ for all measurements.

Hormone analyses

Mean serum concentrations of FSH in mares exhibiting ovulatory cycles during the study were not affected by treatment (Figure 3), but differed due to day of the cycle ($P < 0.10$). Overall, FSH values during estrus were lower than during all other days of the estrous cycle, but did not differ ($P < 0.05$) from values on the day of ovulation. Control mares had higher ($P < 0.05$; Figure 4) levels of LH during all days of the estrous cycle except days 5-8 and 9-12. Moreover, mean LH on the day of ovulation was greater ($P < 0.05$) in control (2.57 ± 0.4 ng/ml) than in GnRH-treated (1.32 ± 0.5 ng/ml) mares. Mean serum concentrations of progesterone were also higher ($P < 0.10$; Figure

5) in control mares than GnRH-treated mares during days 1-4 and 9-12 with higher values observed in control mares.

Mean serum concentrations of LH and FSH for mares which had entered anovulation during the study, did not differ between control and GnRH-treated mares. However, some differences ($P < 0.05$) were observed in mean concentrations of LH and FSH between weeks (Figure 6) and between months for FSH (Figure 7) in anovulatory mares. During week 21, mean concentrations of LH were the lowest (0.09 ± 0.01 ng/ml) and began to rise steadily, reaching 0.3 ng/ml by the end of the experiment at week 28. Mean concentrations of FSH in anovulatory mares reached their lowest value (4.54 ± 0.2 ng/ml) during week 8, and remained low until week 17, at which point concentrations steadily increased to greater than 6.5 ng/ml. Mean serum concentrations of FSH in anovulatory mares during November, December and January were lower ($P < 0.05$; Figure 7) than those measured during September, October, February and March.

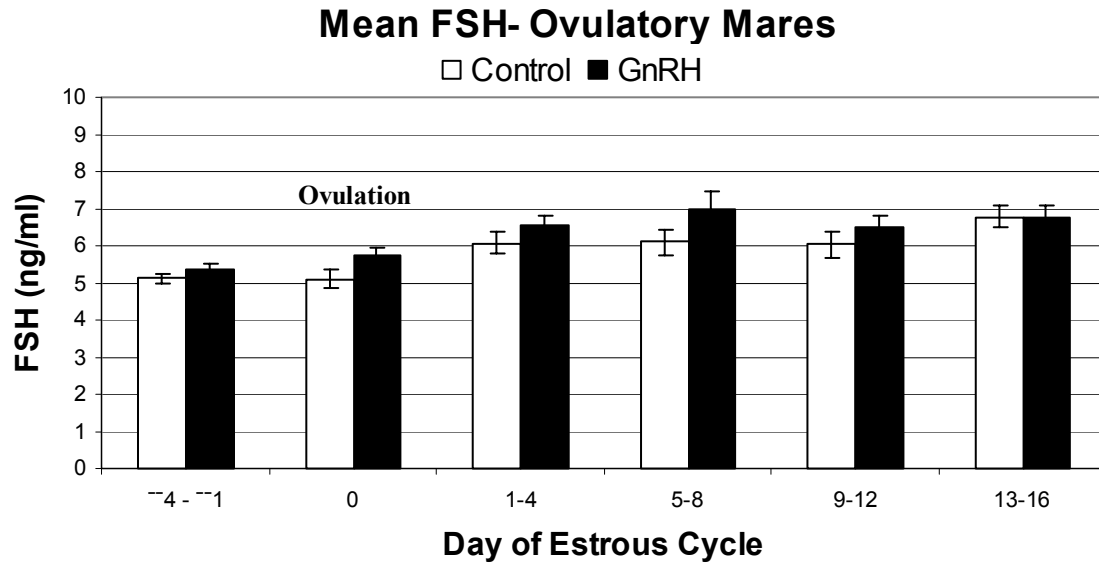


Figure 3: Mean serum concentrations of FSH (ng/ml \pm SEM) in mares exhibiting ovulatory cycles at the beginning and/or the end of the study, relative to day of the estrous cycle. Means did not differ between treatments.

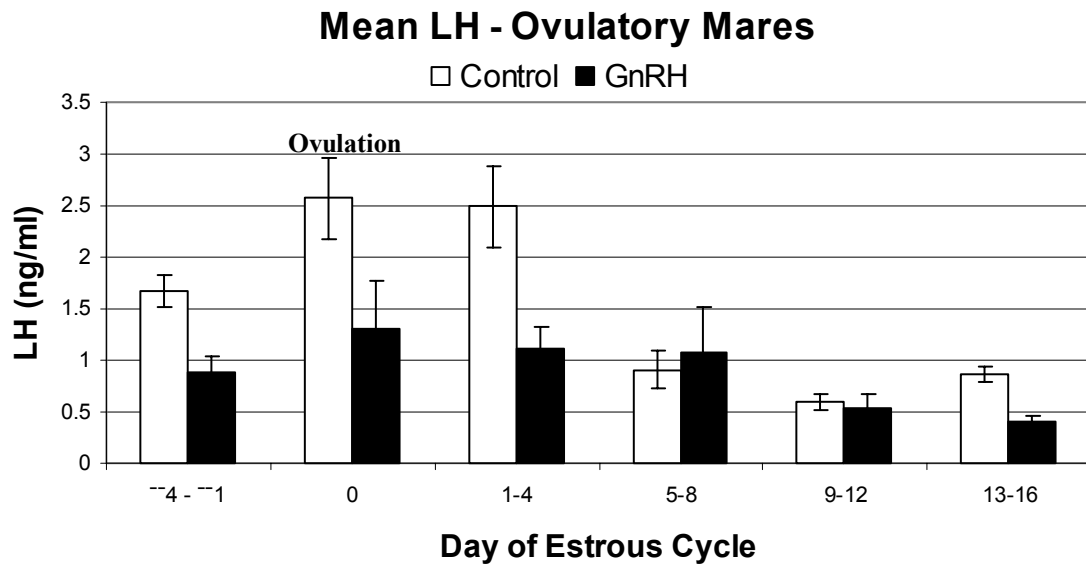


Figure 4: Mean serum concentrations of LH (ng/ml \pm SEM) in mares exhibiting ovulatory cycles at the beginning and/or end of the study, relative to day of the estrous cycle. *Means differed between treatment groups ($P < 0.05$).

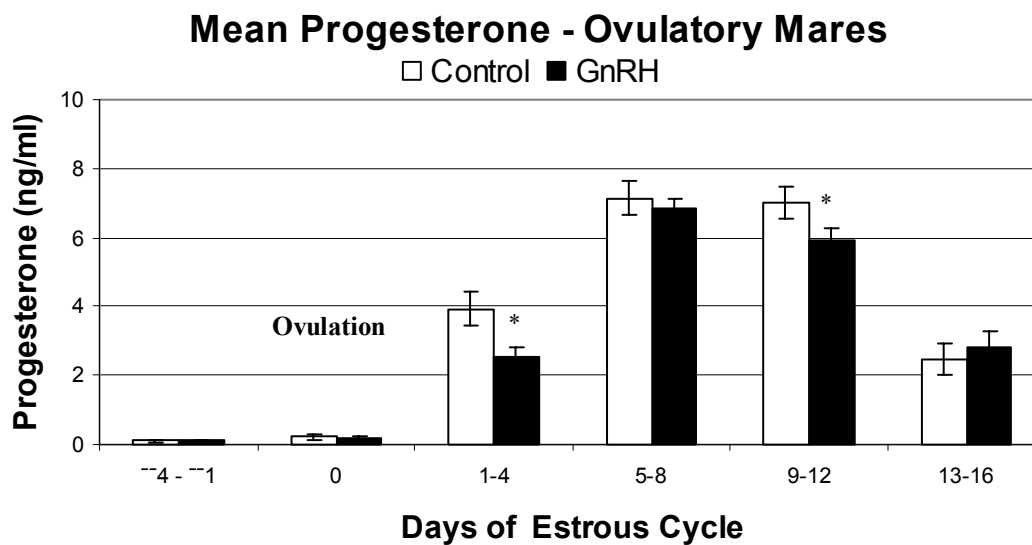


Figure 5: Mean serum concentrations of progesterone (ng/ml \pm SEM) in mares exhibiting ovulatory cycles at the beginning and/or end of the study, relative to day of the estrous cycle. *Means differed between treatment groups ($P < 0.10$).

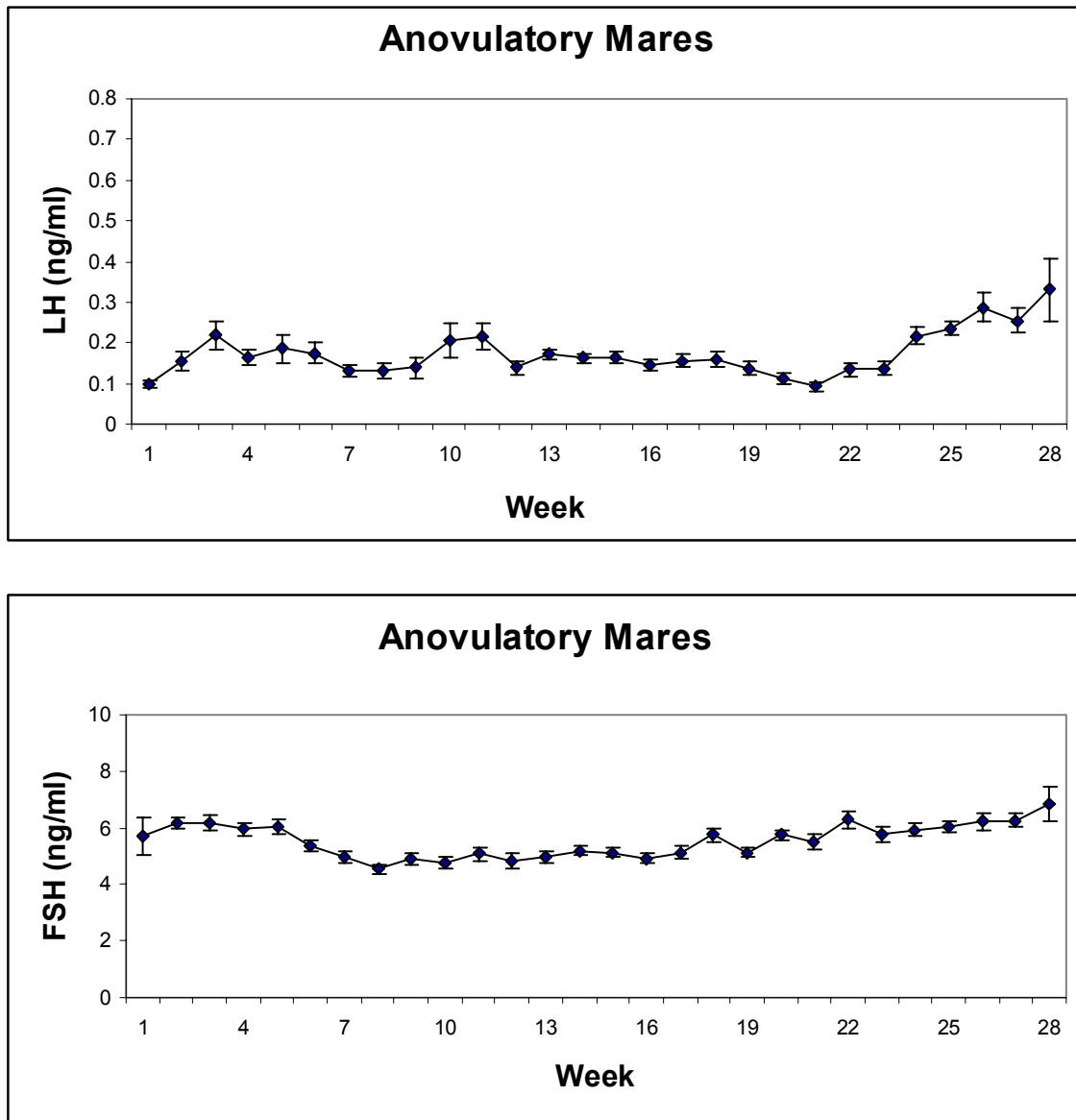


Figure 6: Mean serum concentrations of LH and FSH (ng/ml \pm SEM) in anovulatory mares throughout the 28-week experiment. Data represent pooled mean values from both treatment groups, as mean serum concentrations did not differ between groups.

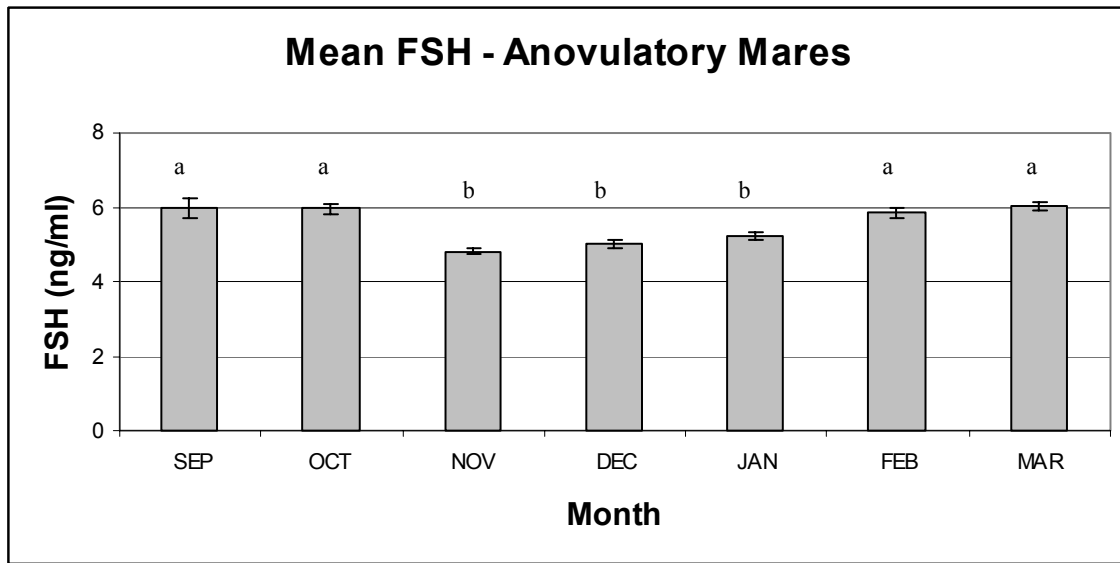


Figure 7: Pooled mean concentrations of FSH (ng/ml \pm SEM) in anovulatory control and GnRH-treated mares averaged over each month of the study. ^{a,b} Means with different superscripts differ ($P < 0.05$).

Anterior pituitary responses to a single intravenous injection of GnRH

Mean plasma concentrations of LH and areas under the curve following intravenous administration of 1.0 mg GnRH were similar for control and GnRH pump-treated mares (Figure 8). Maximal release of LH following injection was achieved within 45 min post-injection in all mares. Peak concentrations of LH ranged from 0.41 to 1.17 ng/ml. Mean baseline and peak values of LH were 0.19 ± 0.07 and 0.79 ± 0.15 ng/ml, respectively, for control and 0.23 ± 0.01 and 0.68 ± 0.14 ng/ml, respectively for GnRH-treated mares. Serum concentrations did not return to baseline by the end of the 5 h experiment.

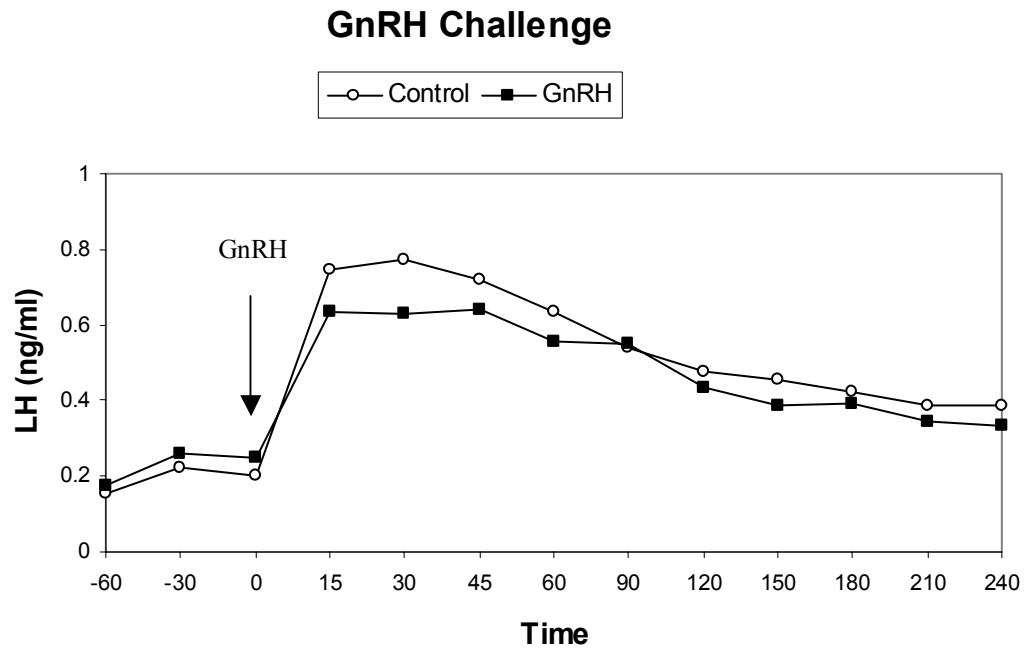


Figure 8: Mean serum concentrations of LH after a 1.0 mg iv. injection of GnRH in a subgroup of control (n=5) and GnRH-treated (n=5) mares. Mean baseline concentrations, peak concentrations, and areas under the curve did not differ between groups.

CHAPTER IV

CONCLUSIONS

Results of this experiment indicate that sc continuous infusion of GnRH at rates of 2.5 and 5.0 ug/h, are too low to provide enough pituitary stimulation for maintaining adequate gonadotropin secretion, follicular growth, and ovulation throughout the non-breeding season. Previous experiments conducted in this laboratory employed GnRH at a rate of 2.5 ug/h to induce follicular development in mares with idiopathic or lactational anovulation during the operational breeding season (37). Approximately 85% of mares responded within 42 days of treatment. Additionally, adverse effects on circulating gonadotropin concentrations and interovulatory intervals were not observed when the same treatment was applied to a limited number of cyclic mares during the breeding season (37). Results of the current study imply that the doses of GnRH administered were inadequate to overcome the lack of photoperiodic stimulation and low endogenous secretion of GnRH.

In the current study, mean concentrations of FSH and progesterone in ovulatory, GnRH-treated mares demonstrated a pattern of secretion typical of the estrous cycle. However, both groups exhibited maximal concentrations of LH before or on the day of ovulation rather than after ovulation as reported previously (1). Additionally, values were lower than those expected during the natural breeding season when endogenous GnRH concentrations are higher. McCue et al. also observed peak circulating concentrations of LH coinciding with the day of ovulation in seasonally anestrous mares

treated with GnRH (10 ug/h) or GnRHa (10 ug sc twice daily) during the anovulatory season (8). Additionally, mares treated with GnRH (7.4 ± 1.5 ng/ml) had significantly higher ($P < 0.05$) concentrations of LH on the day of ovulation than GnRHa-treated (1.8 ± 0.2 ng/ml) mares (8). During the period when most mares were anovulatory, mares that continued to ovulate exhibited peak concentrations of LH on the day of ovulation as opposed to the days following ovulation as reported previously (1). The findings from this study and that of McCue et al. indicate that follicles can undergo final maturation and spontaneous ovulation during the winter months with much lower levels of circulating LH. However, during the breeding season, ovulation most likely occurs prior to the presence of maximal LH concentrations due to overall higher levels of circulating gonadotropins and the extended half-life of equine LH. The increased concentrations during the breeding season may also be accounted for by elevated concentrations of estrogen and enhanced pituitary responsiveness.

Differences between months in mean concentrations of FSH in anovulatory mares (Figure 7) were relatively small. However, these differences are in contrast to previous reports indicating that FSH release does not fluctuate in response to seasonal changes (1, 13). Changes in mean FSH, while likely reflecting a seasonal influence, could also reflect differences between mares in their rate of transition into anovulation and during follicular resurgence (1). Observed differences between weeks in mean concentrations of LH in anovulatory control mares were expected. However, mean concentrations of LH were low for anovulatory mares in both treatment groups near the start of the experiment and continued to decline through the winter, reflecting limited

photoperiodic stimulation, reduced endogenous GnRH secretion, and inadequate exogenous GnRH stimulation in GnRH-treated mares. Additionally, the gradual increase in mean LH in all mares after week 23 paralleled an increase in follicular activity typical of spring transition.

Mean concentrations of LH for mares in this study are generally much lower than those reported in the literature (5, 34, 38, 39) for mares in various reproductive stages. The basis for this resides within our use of a highly specific, homologous RIA in the current study compared to the use of heterologous assays employing relatively impure preparations as references in earlier reported studies. The current assays for LH and FSH produced values that were similar to the homologous and heterologous assays reported by McShan and Papkoff (1, 7, 40, 41)

Pulses of GnRH in the mare have been reported to be secreted at low amplitudes in a virtually continuous manner with occasional high amplitude pulses (22-24). Large pulses of GnRH are associated with concurrent pulses of LH and FSH, but low amplitude pulses of GnRH are not always associated with detectable increases in gonadotropin secretion. Irvine and Alexander demonstrated that about 35% of low amplitude pulses of GnRH are not associated with a pulse of LH or FSH during the luteal phase (23). A similar study in mares during the periovulatory period reported that 9% of low amplitude GnRH pulses failed to initiate a pulse of LH or FSH (24). In other species such as the cow and ewe, low amplitude pulses of GnRH stimulate gonadotropin synthesis and storage, while release of these stores requires higher amplitude pulses of GnRH (42-45). In those species, pharmacological doses of GnRH can be used to

estimate total releasable pools of relatively large stores of LH, even during anestrus/anovulation (46). Similarly, at least one report in the mare has demonstrated that the magnitude of GnRH induced LH release is highly correlated with the amount of LH present in the pituitary, but unlike cattle and sheep, pituitary stores in the mare also appear to reflect circulating concentrations of LH as well (38). In the current study, a 1.0-mg injection of GnRH was administered in March to a selected group of control and GnRH pump-treated mares, to determine if continuous administration of GnRH had affected pituitary synthesis and storage of LH. At this time, mean serum concentrations of LH were extremely low, with most values at the detection limit of the assay for a majority of mares. Results indicated that continuous low-dose treatment with GnRH failed to adequately stimulate gonadotropin synthesis and storage, as releasable pools of LH were low and typical of the anovulatory season. Therefore, our results confirm that releasable pools of LH are highly correlated with mean circulating concentrations in the mare. However, the fact that there was no differences in anterior pituitary responses to GnRH between control and GnRH pump groups indicates that GnRH receptors were not down-regulated in GnRH pump-treated mares. If down-regulation of GnRH receptors were occurring in GnRH pump-treated mares, a lower response to the GnRH challenge would have been likely demonstrated in these mares compared to controls.

In contrast to anovulatory mares, some evidence emerged during the current study that continuous GnRH treatment had down-regulated GnRH receptor populations in ovulatory mares. Mean concentrations of LH were lower in ovulatory, GnRH-treated mares than in control mares, during the estrous cycles that were recorded in late

fall/early winter and in the spring. However, these reduced levels did not affect follicular development, ovulation frequency, or interovulatory intervals. Becker and Johnson reported that cyclic mares receiving continuous infusion of GnRH at a rate of 20 ug/h had lower serum concentrations of LH on the day of ovulation compared to mares treated with GnRH in a pulsatile manner and control groups (10). However, mean concentration of LH in mares receiving pulsatile infusions of GnRH did not differ from controls in that study. Additionally, mean days to ovulation did not differ between treatments (10). Continuous treatment with GnRH in the current study also did not affect interovulatory intervals or the number of ovulations. Thus, while some down-regulation of the GnRH receptor may have occurred in ovulatory mares, coincident with a reduction in mean LH, no effects on estrous cycle events could be documented. Nonetheless, if the interpretation that GnRH receptor down-regulation could account for the lower circulating LH in GnRH-treated mares is correct, it is in contrast to other reports. Porter and Sharp observed increased GnRH receptor binding in vitro in the presence of continuous GnRH infusion (4). Other studies demonstrate that GnRH receptors are continually present throughout the physiological non-breeding season and do not exhibit seasonal changes in concentration (4, 13). When acyclic mares were continuously infused with 25 ug/h of GnRH, pulses of LH occurred twice every 8 h (40). Alexander and Irvine (21) suggest that the combined endogenous and exogenous effects of GnRH in anovulatory mares can be raised to an effective level which stimulates a pulsatile secretory pattern of LH and FSH.

In summary, although continuous administration of GnRH at rates of 2.5 ug/h and 5.0 ug/h did not prevent seasonal anovulation, they also did not appear to interfere with follicular development and ovulation during ovulatory cycle. Observations of estrous cycles beyond 60 days of treatment onset and resumption of some cycles in March, paralleling the natural breeding season, would support this contention. It is possible that a regimen similar to the current one but at higher doses of GnRH could be effective in preventing seasonal anovulation. Notwithstanding economic considerations, successful development of such a strategy could potentially reduce the need for other less effective managerial methods utilized during spring transition into the breeding season. The low mean concentration of LH in ovulatory mares treated with continuous GnRH suggested that some interference with the hypothalamic-pituitary axis of these mares might be occurring. However, GnRH receptor down-regulation as a basis of this phenomenon is not supported by other reported studies.

REFERENCES

1. Ginther OJ. Reproductive Biology of the Mare; Basic and Applied Aspects, Cross Plains, WI: Equiservices, 1992, pp. 105-172, 233-290.
2. Fitzgerald BP, Affleck KJ, Barrows SP, Murdoch WL, Barker KB and Loy RG. Changes in LH pulse frequency and amplitude in intact mares during the transition into the breeding season. *J Reprod Fert* 1987;79:485-491.
3. Malinowski K, Johnson AL and Scanes CG. Effects of interrupted photoperiods on the induction of ovulation in anestrus mares. *J Anim Sci* 1985;61:951-955.
4. Porter MB and Sharp DC. Gonadotropin-releasing hormone receptor trafficking may explain the relative resistance to pituitary desensitization in mares. *Theriogenology* 2002;58:523-526 (abstr.).
5. Silvia PJ, Squires EL and Nett TM. Changes in the hypothalamic-hypophyseal axis of mares associated with seasonal reproductive recrudescence. *Biol Reprod* 1986;35:897-905.
6. Loumaye E, Naor Z and Catt KJ. Binding affinity and biological activity of gonadotropin-releasing hormone agonists in isolated pituitary cells. *Endocrinology* 1982;111:730-736.
7. Turner JE and Irvine CHG. The effect of various gonadotropin-releasing hormone regimens on gonadotrophins, follicular growth and ovulation in deeply anestrus mares. *J Reprod Fert* 1991;44:213-255 (suppl.).

8. McCue PM, Troedsson MHT, Liu IKM, Stabenfeldt GH, Hughes JP and Lasley BL. Follicular and endocrine responses of anoestrous mares to administration of native GnRH or a GnRH agonist. *J Reprod Fert* 1991;44:227-223 (suppl.).
9. Johnson AL. Gonadotropin-releasing hormone treatment induces follicular growth and ovulation in seasonally anestrous mares. *Biol Reprod* 1987;36:1199-1206.
10. Becker SE and Johnson AL. Effects of gonadotropin-releasing hormone infused in a pulsatile or continuous fashion on serum gonadotropin concentrations and ovulation in the mare. *J Anim Sci* 1992;70:1208-1215.
11. Irvine CHG, Alexander SL and McKinnon AO. Reproductive hormone profiles in mares during the autumn transition as determined by collection of jugular blood at 6 h intervals throughout ovulatory and anovulatory cycles. *J Reprod Fert* 2000;118:101-109.
12. Palmer E and Guillaume D. Photoperiodism in the equine species – what is a long night? *Anim Reprod Sci* 1992;28:31-38.
13. Hart PJ, Squires EL, Imel KJ and Nett TM. Seasonal variation in hypothalamic content of gonadotropin-releasing hormone (GnRH), pituitary receptors for GnRH, and pituitary content of luteinizing hormone and follicle-stimulating hormone in the mare. *Biol Reprod* 1984;30:1055-1062.
14. Senger PL. *Pathways to Pregnancy and Parturition*, Pullman, WA: Current Conceptions, Inc., 1999, pp. 116-129.

15. Blanchard TJ, Varner DD and Schumacher J. Manipulation of Estrus in the Mare.
In: Manuel of Equine Reproduction, Pratt PW, Merchant T, Roseman H (eds), St. Louis,
MO: Mosby Inc., 1998, pp. 15-24.
16. Clayton RN. Gonadotropin-releasing hormone: its actions and receptors. *J*
Endocrinology 1984;120:11-19.
17. Schoenemann HM, Brown JL and Reeves JJ. LHRH receptor, LH and FSH
concentration in anterior pituitaries of cycling, noncycling, and early pregnant heifers. *J*
Anim Sci 1985;60:532-536.
18. Tortonese DJ, Gregory SJ, Eagle RC, Sneddon CL, Young CL and Townsend J. The
equine hypophysis: a gland for all seasons. *Reprod Fertil Dev* 2001;13:591-597.
19. Skinner DC and Robinson JE. The pars tuberalis of the ewe: no effect of season or
ovariectomy on the distribution, density or presence of immunoreactive cells. *Cell*
Tissue Res 1996;284:117-123.
20. Watson ED, Pederson HG, Thomson SRM and Fraser HM. Control of follicular
development and luteal function in the mare: effects of a GnRH antagonist.
Theriogenology 2000;54:599-609.
21. Wise ME, Nieman D, Stewart J and Nett TM. Effect of number of receptors for
gonadotropin-releasing hormone on the release of luteinizing hormone. *Biol Reprod*
1984;31:1007-1013.
22. Alexander SL and Irvine CHG. GnRH secretion in the mare. *Anim Reprod Sci*
1996;42:173-180.

23. Irvine CHG and Alexander SL. Secretory patterns and rates of gonadotropin-releasing hormone, follicle stimulating hormone, and luteinizing hormone revealed by intensive sampling of pituitary venous blood in the luteal phase mare. *Endocrinology* 1993;132:212-218.
24. Alexander SL and Irvine CHG. Secretion rates and short-term patterns of gonadotrophin-releasing hormone, FSH and LH throughout the periovulatory period in the mare. *J Endocrinol* 1987;114:351-362.
25. Porter MB, Cleaver BD, Peltier M, Robinson G, Thatcher WW and Sharp DC. Comparative study between pony mares and ewes evaluating gonadotrophic response to administration of gonadotropin-releasing hormone. *J Reprod Fert* 1997;110:219-229.
26. Skaggs CL, Able BV and Stevenson JS. Pulsatile or continuous infusion of luteinizing hormone-releasing hormone and hormonal concentrations in prepubertal beef heifers. *J Anim Sci* 1986;62:1034-1048.
27. D'Occhio MJ, Fordyce G, Whyte TR, Aspden WJ and Trigg TE. Reproductive responses of cattle to GnRH agonists. *Anim Reprod Sci* 2000;60-61:433-442.
28. Gong JG, Campbell BK, Bramley TA, Gutierrez CG, Peters AR and Webb R. Suppression in the secretion of follicle-stimulating hormone and luteinizing hormone, and ovarian follicle development in heifers continuously infused with a gonadotropin-releasing hormone agonist. *Biol Reprod* 1996;55:68-74.

29. Gong JG, Bramley TA, Gutierrez CG, Peters AR and Webb R. Effects of chronic treatment with gonadotrophin-releasing hormone agonist on peripheral concentrations of FSH and LH, and ovarian function in heifers. *J Reprod Fert* 1995;105:263-270.
30. D'Occhio MJ, Fordyce G, Whyte TR, Jubb TF, Fitzpatrick LA, Cooper NJ, Aspden WJ, Bolam MJ and Trigg TE. Use of GnRH agonist implants for long-term suppression of fertility in extensively managed heifers and cows. *Anim Reprod Sci* 2002;74:151-162.
31. Kile JP, Alexander BM, Moss GE, Hallford DM and Nett TM. Gonadotropin-releasing hormone overrides the negative effect of reduced dietary energy on gonadotropin synthesis and secretion in ewes. *Endocrinology* 1991;128:843-849.
32. Southworth MB, Matsumoto AM, Gross KM, Soules MR and Bremner WJ. The importance of signal pattern in the transmission of endocrine information: pituitary gonadotropin responses to continuous and pulsatile gonadotropin-releasing hormone. *J Clin Endocrinol Metab* 1991;72:1286-1289.
33. Fitzgerald BP, Meyer SL, Affleck KJ and Silvia PJ. Effect of constant administration of a gonadotropin-releasing hormone agonist on reproductive activity in mares: Induction of ovulation during seasonal anestrus. *Am J Vet Res* 1993;54:1735-1745.
34. Mumford EL, Squires EL, Peterson KD, Nett TM and Jasko DJ. Effect of various doses of gonadotropin-releasing hormone analogue on induction of ovulation in anestrus mares. *J Anim Sci* 1994;72:178-183.

35. Ainsworth CGV and Hyland JH. Continuous infusion of gonadotropin-releasing hormone (GnRH) advances the onset of oestrous cycles in Thoroughbred mares on Australian studfarms. *J Reprod Fert* 1991;44:235-240 (suppl.).
36. Hyland JH and Jeffcott LB. Control of transitional anestrus in mares by infusion of gonadotropin releasing hormone. *Theriogenology* 1988;29:1383-1391.
37. Williams GL, Amstalden M, Garcia MR, Williams SM, Ward J, Blodgett GP and Quirk KS. Control of lactational and idiopathic anovulation in mares with low-dose, native GnRH: Anterior pituitary and ovarian responses to continuous sc infusion. *Biol Reprod* 2000;62:226 (abstr., suppl.).
38. Silvia PJ, Squires EL and Nett TM. Pituitary responsiveness of mares challenged with GnRH at various stages of the transition into the breeding season. *J Anim Sci* 1987;64:790-796.
39. Nett TM, Holtan DW and Estergreen VL. Levels of LH, prolactin and oestrogens in the serum of post-partum mares. *J Reprod Fert* 1975;23:201-206 (suppl.).
40. Hyland JH, Wright PJ, Clarke IJ, Carson RS, Langsford DA and Jeffcott LB. Infusion of gonadotropin-releasing hormone (GnRH) induces ovulation and fertile oestrous in mares during seasonal anoestrus. *J Reprod Fert* 1987;35:211-220 (suppl.).
41. Landefeld TD and McShan WH. Equine luteinizing hormone and its subunits, isolation and physiochemical properties. *Biochemistry* 1974;13:1389-1393.

42. Nett TM, Crowder ME and Wise ME. Role of estradiol in inducing an ovulatory-like surge of luteinizing hormone in sheep. *Biol Reprod* 1984;30:1208-1215.
43. Levine JE, Pau KF, Ramirez VD and Jackson GL. Simultaneous measurement of luteinizing hormone-releasing hormone and luteinizing hormone released in unanesthetized, ovariectomized sheep. *Endocrinology* 1982;111:1449-1455.
44. Clarke IJ and Cummins JT. The temporal relationship between gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) secretion in ovariectomized ewes. *Endocrinology* 1982;111:1737-1739.
45. Gazal OS, Leshin LS, Stanko RL, Thomas MG, Keisler DH, Anderson LL and Williams GL. Gonadotropin-releasing hormone secretion into third-ventricle cerebrospinal fluid of cattle: Correspondence with the tonic and surge release of luteinizing hormone and its tonic inhibition by suckling and neuropeptide Y. *Biol Reprod* 1998;59:676-683.
46. Crowder ME, Giles PA, Tamanini C, Moss GE and Nett TM. Pituitary content of gonadotropins and GnRH-receptors in pregnant, postpartum and steroid treated ovx ewes. *J Anim Sci* 1982;54:1235-1242.

APPENDIX

Progesterone RIA

Single Antibody RIA Kit, Diagnostic Products Corporation, Los Angeles, CA

References:

- Jones, E.J., Armstrong, J.D. and Harvey, R.W. J. Anim. Sci. 69:1607- (1991)
 Diagnostic Products Corporation Coat-A-Count Progesterone Kit, Los Angeles, CA
 Simpson, R.B., Armstrong, J.D. and Harvey, R.W. J. Anim. Sci. 70:1478- (1992).

1. Iodinated Product: Iodination grade hP4.
2. Antibody: Anti-human P4 coated tubes.
3. Standards: Human serum with added P4. Range: 0.1 – 20.0 ng/ml.
4. Reference: Human standard preparation added to bovine serum.
5. RIA Procedure:
 - A. Begin and complete assay
 1. Pipette in non-coated polypropylene tubes
 NSB – 100 µl of 0 std
 2. Pipette in antibody coated tubes
 0 Std – 100 µl
 Std – 100 µl
 Ref – 100 µl
 Unknowns – 100 µl
 3. Pipette 1 ml of ¹²⁵I-P4 provided in the kit into all tubes including two Total Count non-coated polypropylene tubes.
 4. Vortex tubes briefly and incubate at room temperature for 3 h.
 5. Pour off supernatant.
 6. Count radioactivity of each tube using a gamma counter.

Equine FSH RIA

References:

- A.F. Parlow, NIDDK
Williams, G.L., Kotwica, J., Slanger, W.D., Olson, D.K., Tilton, J.E. and
Johnson
L.J. J. Anim. Sci. 54:594- (1982)

1. Iodinated Product: Iodination grade eFSH (AFP-5022B).
2. Antibody: Anti-equine FSH (AFP-2062096). Dilution 1:12,500.
3. Standards: Iodination grade eFSH (AFP-5022B). Range: 0.5 – 25.0 ng/ml.
4. References: eFSH added to equine serum.
5. RIA Procedure:
 - A. Day 1: Begin Assay
 1. NSB – 500 µl of 1% PBS-EW (egg white).
 2. 0 Std – 500 µl of 1% PBS-EW.
 3. Stds – 200 µl std + 300 µl of 1% PBS-EW.
 4. Ref – 200 µl ref + 300 µl of 1% PBS-EW.
 5. Unknown – 200 µl sample + 300 µl of 1% PBS-EW.
 6. Pipette 200 µl of PBS-EDTA + 1:400 NRS without primary antibody into NSB tubes only.
 7. Pipette 200 µl of anti-eFSH (diluted in PBS-EDTA+1:400 NRS) into all tubes except NSB and TC tubes.
 8. Vortex tubes briefly and incubate for 1 hour at room temperature.
 9. Pipette 100 µl ¹²⁵I-eFSH (20,000cpm/100 µl diluted in 0.1% PBS-EW) to all tubes.
 10. Vortex tubes briefly and incubate for 24 h at 4°C.
 - B. Day 2: Add Second Antibody
 1. Keep all test tubes and reagents on ice during all procedures.
 2. Pipette 200 µl of Sheep-anti-rabbit gamma globulin (SARGG; 2nd Ab) diluted in PBS-EDTA without NRS into all tubes except TC tubes.
 3. Vortex tubes briefly and place in refrigeratory for 48-72 h at 4°C.
 - C. Day 4: Take Off Assay
 1. Keep all test tubes and reagents on ice during all procedures.

2. Add 3.0 ml ice cold PBS (0.01 M; pH 7.0) to all tubes except TC tubes.
3. Centrifuge tubes for 1 h at 4°C at 3600 rpm.
4. Decant supernatant.
5. Count radioactivity of each tube using a gamma counter.

Equine LH RIA

References:

- Davis, S.L., Riechert, L.E. and Niswender, G.D. Biol. Reprod. 4:415- (1971)
 Echternkamp, S.E., Bolt, D.J. and Hawk, H.W. J. Anim. Sci. 42:893- (1976)
 Golter, T.D., Reeves, J.J., O'Mary, C.C., Arimura, A. and Schally, A.V. J. Anim. Sci. 37:123- (1973)
 Niswender, G.D., Riechert, L.E., Midgley, A.R. and Nalbandov, A.V. Endocrinology 84:1166- (1969)
 Williams, G.L. and Ray, D.E. J. Anim. Sci. 50:906- (1980)

1. Iodinated Product: Iodination grade eLH (AFP-5130A).
2. Antibody: Anti-equine LH (AFP-240580). Dilution 1:120,000.
3. Standards: Iodination grade eLH (AFP-5130A). Range: 0.1 – 20.0 ng/ml.
4. References: eLH added to equine serum.
5. RIA Procedure:
 - D. Day 1: Begin Assay
 1. NSB – 500 µl of 1% PBS-EW (egg white).
 2. 0 Std – 500 µl of 1% PBS-EW.
 3. Stds – 200 µl std + 300 µl of 1% PBS-EW.
 4. Ref – 200 µl ref + 300 µl of 1% PBS-EW.
 5. Unknown – 200 µl sample + 300 µl of 1% PBS-EW.
 6. Pipette 200 µl of PBS-EDTA + 1:400 NRS without primary antibody into NSB tubes only.
 7. Pipette 200 µl of anti-eLH (diluted in PBS-EDTA+1:400 NRS) into all tubes except NSB and TC tubes.
 8. Vortex tubes briefly and incubate for 1 hour at room temperature.
 9. Pipette 100 µl ¹²⁵I-eLH (20,000cpm/100 µl diluted in 0.1% PBS-EW) to all tubes.
 10. Vortex tubes briefly and incubate for 24 h at 4°C.
 - E. Day 2: Add Second Antibody
 1. Keep all test tubes and reagents on ice during all procedures.
 2. Pipette 200 µl of Sheep-anti-rabbit gamma globulin (SARGG; 2nd Ab) diluted in PBS-EDTA without NRS into all tubes except TC tubes.

3. Vortex tubes briefly and place in refrigerator for 48-72 h at 4°C.

F. Day 4: Take Off Assay

1. Keep all test tubes and reagents on ice during all procedures.
2. Add 3.0 ml ice cold PBS (0.01 M; pH 7.0) to all tubes except TC tubes.
3. Centrifuge tubes for 1 h at 4°C at 3600 rpm.
4. Decant supernatant.
5. Count radioactivity of each tube using a gamma counter.

VITA

Stephanie Morton

PERMANENT ADDRESS

4613 Pembroke Lane, Bryan, Tx 77802; Phone: (979) 776-0279; email: smorton@cvm.tamu.edu

EDUCATION

B.S., Agriculture (Animal Science major), McNeese State University, Lake Charles, La, 2000

M.S., Physiology of Reproduction, Texas A&M University, College Station, TX, 2004

SELECTED PUBLICATIONS

Journal Articles

Zieba, D.A., M. Amstalden, J.L. Gallino, S. Morton, and G.L. Williams. 2003. Effects of leptin on basal and growth hormone-releasing hormone-stimulated growth hormone secretion from bovine adeno hypophysis are dependent upon nutritional status. *J. Endocrinol.* 178: 83-89

Amstalden, M., D.A. Zieba, J.L. Gallino, S. Morton, J.F. Edwards, P.G. Harms, T.H. Welsh, Jr., R.L. Stanko, D.H. Keisler, and G.L. Williams. 2003. Leptin acts at the bovine adeno hypophysis to enhance basal and GnRH-mediated release of LH: Differential effects are dependent upon nutritional history. *Biol. Reprod.* 69: 1539-1544

Zieba, D.A., M. Amstalden, S. Morton, M.N. Maciel, D.H. Keisler, and G.L. Williams. 2003. Regulatory roles of leptin at the hypothalamic-hypophyseal axis before and after sexual maturation in cattle. *Biol. Reprod.* (**submitted**)

Morton, S., D.A. Zieba, and G.L. Williams. Can continuous treatment with GnRH prevent seasonal anovulation in mares? (**In preparation**)